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Mitochondrial DNA Somatic Mutations (Point Mutations and Large Deletions) and Mitochondrial DNA Variants in Human Thyroid Pathology

A Study with Emphasis on Hürthle Cell Tumors

Valdemar Máximo,* Paula Soares,*† Jorge Lima,* José Cameselle-Teijeiro,‡ and Manuel Sobrinho-Simões*†§

From the Institute of Molecular Pathology and Immunology, the University of Porto, Porto, Portugal; the Department of Pathology,† Medical Faculty of Porto, Porto, Portugal; the Department of Pathology,§ Hospital São João, Porto, Portugal; and the Department of Pathology,‡ Hospital Clínico Universitario, Santiago de Compostela, Spain*

In an attempt to progress in the understanding of the relationship of mitochondrial DNA (mtDNA) alterations and thyroid tumorigenesis, we studied the mtDNA in 79 benign and malignant tumors (43 Hürthle and 36 non-Hürthle cell neoplasms) and respective normal parenchyma. The mtDNA common deletion (CD) was evaluated by semiquantitative polymerase chain reaction. Somatic point mutations and sequence variants of mtDNA were searched for in 66 tumors (59 patients) and adjacent parenchyma by direct sequencing of 70% of the mitochondrial genome (including all of the 13 OXPHOS system genes). We detected 57 somatic mutations, mostly transitions, in 34 tumors and 253 sequence variants in 59 patients. Follicular and papillary carcinomas carried a significantly higher prevalence of nonsilent point mutations of complex I genes than adenomas. We also detected a significantly higher prevalence of complex I and complex IV sequence variants in the normal parenchyma adjacent to the malignant tumors. Every Hürthle cell tumor displayed a relatively high percentage (up to 16%) of mtDNA CD independently of the lesion's histotype. The percentage of deleted mtDNA molecules was significantly higher in tumors with D-loop mutations than in mtDNA stable tumors. Sequence variants of the *ATPase 6* gene, one of the complex V genes thought to play a role in mtDNA maintenance and integrity in yeast, were significantly more prevalent in patients with Hürthle cell tumors than in patients with non-Hürthle cell neoplasms. We conclude that mtDNA variants and mtDNA somatic mutations of complex I and complex IV genes seem to be involved in thyroid tumorigenesis. Germline polymorphisms of the *ATPase 6* gene are associated with the occurrence of mtDNA CD, the hallmark of Hürthle cell tumors. ***(Am J Pathol 2002, 160:1857–1865)***

Hürthle (oxyphil) cells are found in a minority of thyroid tumors, either benign (Hürthle cell adenoma) or malignant (Hürthle cell variants of follicular and papillary carcinoma), as well as in other types of thyroid tumors and several nonneoplastic thyroid disorders.[1,2] Hürthle cells are characterized by a large, granular, eosinophilic cytoplasm, which is filled with abnormal mitochondria. Most Hürthle cell tumors are sporadic and frequently occur in association with autoimmune thyroiditis, but their occurrence in a familial setting has also been reported.[3,4] The abundance of abnormal mitochondria makes Hürthle cell tumors a good model to study mtDNA abnormalities in human cancer.

Mitochondrial DNA (mtDNA) is thought to be more susceptible than nuclear DNA to mutagen-induced damage for several reasons: mtDNA polymerase γ replicates the DNA with poor fidelity,[5] mtDNA is a naked (without histones) molecule to which chemical carcinogens can easily bind,[6,7] and mtDNA is particularly susceptible to the high concentration of reactive oxygen species in mitochondria.[8] Nuclear microsatellite instability (nMSI) is related to functional loss of mismatch repair genes, including the *hMSH2*, *hMLH1*, *hPMS1*, and *hPMS2* genes.[9,10] In the mitochondrial genome, the mismatch repair system has been found only in yeast strains in which MSH1 and MSH2 are separately involved in mitochondrial and nuclear DNA repair systems, respectively.[11] No MSH1 homologue has been found in mammalian cells and it remains uncertain whether a mismatch repair system plays a role in the maintenance of the mammalian mitochondrial genome.

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Address reprint requests to Dr. M. Sobrinho-Simões, IPATIMUP, Rua Dr. Roberto Frias s/n, 4200-465 Porto, Portugal. E-mail: ssimoes@ipatimup.pt.

The term mitochondrial microsatellite instability (mtMSI) was introduced by Habano and colleagues,[12] in a study on colorectal tumors, to describe alterations in repetitive regions of mtDNA. For the evaluation of mtMSI, Habano and colleagues[12] quantified the alterations in two simple repeat sequences in a noncoding displacement-loop (D-loop) region in mtDNA. Additional studies have addressed the issue of mtMSI in human cancers[13–17] without reaching concordant conclusions about the relationship between the instability of nuclear and mitochondrial genomes.[12–17]

Alterations of mtDNA have been demonstrated in various types of human cancer and include large deletions, missense mutations, frameshift mutations, and small deletions/insertions.[1,12–26] mtDNA is a hot spot for mutations in cancer as it is preferentially damaged by many carcinogens.[6,7] The role of mtDNA somatic mutations in this setting is not yet understood.[14,21–23]

We have previously detected the mitochondrial common deletion (mtDNA CD) in a small series of thyroid tumors composed of Hürthle (oxyphil) cells, as well as in some nonneoplastic thyroid lesions with incipient Hürthle cell changes.[18,24] The mtDNA CD has also been detected in Hashimoto's thyroiditis displaying oxyphilic cells.[1]

Very few studies analyzing mtDNA mutations in thyroid have been published to date.[1,18,23,24,26,27] Such studies were limited by the small size of the samples and the small percentage of mtDNA analyzed per case.[1,18,23,24,26,27] In an attempt to progress in the understanding of the putative relationship between mtDNA alterations in thyroid tumors in general, and Hürthle cell tumors in particular, we searched for mtDNA alterations in a large series of thyroid tumors, including both benign and malignant lesions, paying a special attention to the different histotypes of Hürthle cell neoplasms. In each case we have also analyzed the mtDNA of normal adjacent parenchyma in an attempt to find sequence variants of mtDNA putatively associated with the occurrence of Hürthle cell tumors.

Materials and Methods

Materials

Seventy-nine thyroid tumors from 68 patients were studied. In 11 patients there were two distinct lesions that were separately studied. The 79 lesions were classified according to Hedinger and colleagues[28] and Rosai and colleagues[29] as follicular adenoma ($n = 15$), follicular Hürthle cell adenoma ($n = 20$), follicular carcinoma ($n = 5$), follicular Hürthle cell carcinoma ($n = 13$), papillary carcinoma ($n = 16$), and papillary Hürthle cell carcinoma ($n = 10$). Samples from 32 lesions were obtained at the time of surgery, together with the corresponding normal adjacent tissues; these samples were carefully dissected by expert pathologists and snap-frozen. In 47 cases, microdissected paraffin-embedded material was used for the screening of mtDNA mutations because of the absence of representative tumor tissue in the frozen samples.

DNA Extraction

DNA was extracted from microdissected frozen and/or paraffin-embedded pathological and normal thyroid tissue pairs using the NucleoSpin Tissue Kit (Macherey-Nagel, Düren, Germany).

Screening of mtDNA CD by Polymerase Chain Reaction (PCR)

The detection of mtDNA CD was performed using two sets of primers: Mitout-F and Mitout-R (outside the deletion region) and Mitin-F and Mitin-R (within the deletion region).[16,18] In the wild-type mtDNA only the Mitin primer set gives a PCR product with 142 bp. In cases with the mtDNA CD, Mitin primers amplify a 142-bp target sequence and Mitout primers an aberrant PCR product with 214 bp.[16] PCR amplifications were performed in a 25-μl volume containing 200 μmol/L of each dNTP, 12.5 pmol of each of the forward and reverse primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl, (pH 9.0), 1.5 mmol/L $MgCl_2$, and 1 U of *Taq* DNA polymerase (Amersham Biosciences, Lda, Buckinghamshire, England). Cycling conditions were a single predenaturation step at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 20 seconds, annealing at 60°C for 20 seconds, and elongation at 72°C for 20 seconds, and a final incubation at 72°C for 2 minutes. PCR products were inspected by electrophoresis on 2% agarose gels.

Semiquantitative PCR

For the quantitation of the percentage of mtDNA molecules deleted in each sample, PCR co-amplification of two fragments of mtDNA (one within and the other outside the deletion region) were performed. PCR co-amplifications were performed in a 25-μl volume containing 200 μmol/L of each dNTP, 12.5 pmol of each of the forward and reverse of both sets of primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl, (pH 9.0), 1.5 mmol/L $MgCl_2$, and 1 U of *Taq* DNA polymerase (Amersham Biosciences, Lda). Cycling conditions were a single predenaturation step at 94°C for 5 minutes followed by 18 cycles of denaturation at 94°C for 20 seconds, annealing at 62°C for 20 seconds, and elongation at 72°C for 20 seconds, and a final incubation at 72°C for 2 minutes. PCR products were inspected by electrophoresis on 2% agarose gels. The optimal number of cycles of amplification to allow quantitation of the two PCR products was determined using three samples of normal thyroid. Two hundred ng of each DNA sample were subjected to a number of amplification cycles ranging from 10 to 25 cycles. PCR products were separated on a 2% agarose gel and stained with ethidium bromide. The intensity of the fluorescence was automatically measured and integrated with the genescan software Image Master (Amersham Biosciences, Lda). A close to exponential increase in the amount of PCR product was obtained between 15 and 23 cycles for both fragment products. In every semiquantitative PCR experiment 18 cycles were used. The determination of the

optimal annealing temperature of the two sets of primers was performed using the same three samples of DNA of normal thyroid samples used in the determination of the optimal number of cycles for amplification. PCR triplicates of 200 ng of DNA of each sample were used for co-amplification of both fragments. Cycling conditions were a single predenaturation step at 94°C for 5 minutes followed by 18 cycles of denaturation at 94°C for 20 seconds, annealing varying between 54°C and 65°C for 20 seconds, and elongation at 72°C for 20 seconds, and a final incubation at 72°C for 2 minutes. PCR products were inspected by electrophoresis on 2% agarose gels. At 62°C the amount of PCR products of both fragments was similar. In all quantitation analyses, 18 cycles of PCR amplification and an annealing temperature of 62°C were used.

Screening of mtDNA Somatic Mutations and mtDNA Variants by Direct Sequencing

By PCR/direct sequencing we analyzed 66 thyroid tumors and the respective adjacent normal thyroid tissue, surgically excised from 59 patients. In the remaining 13 tumors the study could not be performed for technical reasons. In seven cases (patients 52 to 58) blood samples were also analyzed. Using fragments varying from 0.6 to 1.4 kb we have screened 70% of the mitochondrial genome: all mtDNA coding genes, 46% of tRNA genes ($tRNA^{Phe, Gly, Lys, Asp, Leu1, Ser1, His, Leu2, Ile, Ser2, Glu, Arg}$) and 52% of D-loop region. Details of PCR primers and sequencing primers are available on request from the authors. All PCR amplifications were performed in a 25-μl volume containing 200 μmol/L of each dNTP, 12.5 pmol of each of the forward and reverse primers, 50 mmol/L KCl, 10 mmol/L Tris-HCl, (pH 9.0), 1.5 mmol/L $MgCl_2$, and 1 U of *Taq* DNA polymerase (Amersham Biosciences Lda). Cycling conditions were a single predenaturation step at 94°C for 5 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 58°C for 20 seconds, and elongation at 72°C for 1 minute, and a final incubation at 72°C for 5 minutes. PCR products were separated by electrophoresis on 2% agarose gels and purified using the NucleoSpin Extract Kit (Macherey-Nagel, Düren, Germany). Sequencing analysis was then performed on purified products using the ABI Prism dGTP BigDye Terminator Ready Reaction Kit (Perkin-Elmer, Foster City, CA) and an ABI Prism 377 DNA sequencer (Perkin-Elmer). Both strands were screened using the original primers. Sequences were compared against a comprehensive mitochondrial databank (MITOMAP: A Human Mitochondrial Genome Database. Center for Molecular Medicine, Emory University, Atlanta, GA. *http://www.gen.emory.edu/mitomap.html, 2000*). All mtDNA-altered samples were subjected to an additional complete analysis.

Statistical Analysis

The statistical analysis of the results was performed using the chi-square test with the Yates correction, Fisher's exact test, and Student's *t*-test. A nonparametric test (Mann-Whitney) was also used whenever appropriate. A *P* value $<$0.05 was considered statistically significant.

Table 1. Screening and Evaluation of the mtDNA CD in Seventy-Nine Tumors and in the Corresponding Adjacent Parenchyma*

Diagnosis	No. of cases	Percentage of cases showing the mtDNA CD, %	Percentage of deleted mtDNA (mean ± SD), %
AP	15	0	0
A	15	33.3	0.5 ± 0.7
AP	20	25.0	0.3 ± 1.0
HCA	20	100	4.3 ± 1.6
AP	5	0	0
FC	5	0	0
AP	12	33.3	0.3 ± 0.4
HCFC	13	100	7.4 ± 2.2
AP	16	12.5	0.04 ± 0.2
PC	16	18.8	0.2 ± 0.3
AP	9	33.3	0.1 ± 0.3
HCPC	10	100	4.3 ± 1.6

*In two cases there was no adjacent parenchyma available for study.
AP, Adjacent parenchyma; A, adenoma; HCA, Hürthle cell adenoma; FC, follicular carcinoma; HCFC, Hürthle cell follicular carcinoma; PC, papillary carcinoma; HCPC, Hürthle cell papillary carcinoma.

Results

MtDNA CD Screening

The overall results are summarized in Table 1. The mtDNA CD was found in all Hürthle cell tumors (100%, $n = 43$), in 5 of 15 (33.3%) adenomas, and in 3 of 16 (18.8%) papillary carcinomas without Hürthle cell features. The occurrence of mtDNA CD was significantly associated ($P < 0.001$) with Hürthle cell tumors. The mtDNA CD was also detected at very low levels in the normal adjacent thyroid tissue of some cases (Table 1). The histological study of the specimens in which mtDNA CD was observed, including the positive peritumoral tissues, revealed either typical Hürthle cells or follicular cells with relatively abundant, granular, oxyphilic cytoplasm of a kind we have previously designated as incipient Hürthle cell transformation.[2,18]

Percentage of mtDNA CD

The average percentage and the SD of mtDNA CD in the lesions of each group are summarized in Table 1. The amount of mtDNA CD was significantly higher ($P <$ 0.0001) in Hürthle cell tumors than in non-Hürthle cell tumors independently of the benignity or malignancy of the lesions. The percentage of mtDNA CD was significantly higher ($P < 0.0001$ and $P = 0.0012$, respectively) in Hürthle cell follicular carcinomas (7.4 ± 2.2%) than in Hürthle cell adenomas (4.3 ± 1.6%) and Hürthle cell papillary carcinomas (4.3 ± 1.6%) (Table 1).

MtDNA Somatic Point Mutations

D-Loop Region

The results of the screening of D-loop somatic mutations are summarized in Table 2. Most of the mutations

Table 2. Summary of mtDNA D-Loop Alterations in Sixty-Six Tumors

Case	Age	Diagnosis	Nucleotide position	Nucleotide change
1	57	PC	514	$CA_7 \rightarrow CA_4$
2	51	HCFC	303	$C_6 \rightarrow C_{6,7}$
			497	$C \rightarrow T$
			499	$G \rightarrow A$
3	32	HCFC	303	$C_6 \rightarrow C_{8,9}$
			325	$C \rightarrow T$
			514	$CA_5 \rightarrow CA_6$
			568	$C_6 \rightarrow C_8$
4	39	HCFC	568	$C_6 \rightarrow C_{8-10}$
5	33	HCFC	460	$T \rightarrow C$
			514	$CA_5 \rightarrow CA_7$
6	76	HCFC	514	$CA_5 \rightarrow CA_6$
7	62	HCPC	303	$C_7 \rightarrow C_8$
			514	$CA_5 \rightarrow CA_6$
9	49	HCA	303	$C_6 \rightarrow C_8$
10A	63	PC	303	$C_7 \rightarrow C_8$
10B	63	HCA	195	$T \rightarrow C$
			303	$C_7 \rightarrow C_8$
12A	45	HCA	303	$C_6 \rightarrow C_8$
			481	$C \rightarrow T$
14	34	HCA	303	$C_7 \rightarrow C_{9,10}$
15	59	HCA	303	$C_7 \rightarrow C_8$
16A	67	HCA	303	$C_7 \rightarrow C_8$
			456	$C \rightarrow T$
16B	67	HCA	303	$C_7 \rightarrow C_{8,9}$
17	57	HCA	462	$C \rightarrow T$
			303	$C_7 \rightarrow C_8$
18	38	HCA	207	$G \rightarrow A$
			303	$C_7 \rightarrow C_{8,9}$
			568	$C_6 \rightarrow C_7$
19	54	HCFC	115	$T \rightarrow C$
			514	$CA_5 \rightarrow CA_6$
21	8	HCPC	185	$G \rightarrow A$
			514	$CA_4 \rightarrow CA_{4-6}$
22	46	HCPC	303	$C_7 \rightarrow C_8$
			514	$CA_5 \rightarrow CA_6$
27	48	HCFC	514	$CA_5 \rightarrow CA_6$
28	42	A	303	$C_7 \rightarrow C_{9,10}$
32	32	HCPC	303	$C_7 \rightarrow C_{9,10}$
39	53	PC	514	$CA_5 \rightarrow CA_8$
			549	$C \rightarrow T$
40	77	HCA	514	$CA_5 \rightarrow CA_{6,7}$
41	40	FC	150	$C \rightarrow T$
			303	$C_7 \rightarrow C_{8-10}$
46	67	A	514	$CA_7 \rightarrow CA_6$
51	54	PC	303	$C_7 \rightarrow C_{8-12}$
			325	$C \rightarrow T$
			514	$CA_5 \rightarrow CA_{6,7}$
53	37	A	98	C del
			514	$CA_4 \rightarrow CA_5$
54	56	HCA	303	$C_7 \rightarrow C_8$
57	34	HCA	73	$A \rightarrow G$
			107	G del
			303	$C_7 \rightarrow C_{8,9}$
60	56	PC	150	$C \rightarrow T$
			514	$CA_5 \rightarrow CA_6$

found in the D-loop region were located in repetitive regions. Following Habano and colleagues[20] we classified the tumors displaying somatic mutations in repetitive regions (variations in the number of repetitive units) as lesions with mtDNA D-loop instability. MtDNA D-loop instability was detected in 32 of 66 (48.5%) tumors (Table 2). By tumor type, mtDNA D-loop instability was found in 3 of 10 (30.0%) adenomas, 12 of 20 (60.0%) Hürthle cell adenomas, 1 of 4 (25.0%) follicular carcinomas, 7 of 13 (53.9%) Hürthle cell follicular carcinomas, 5 of 12 (41.7%) papillary carcinomas, and in 4 of 7 (57.1%) Hürthle cell papillary carcinomas. The percentage of tumors with D-loop instability is similar ($P = 0.822$) in benign (50.0%, 15 of 30) and in malignant tumors (47.2%, 17 of 36). The percentage of Hürthle cell tumors with mtDNA D-loop instability (57.5%, 23 of 40) is higher, although not significantly ($P = 0.069$), than the percentage of non-Hürthle cell tumors with mtDNA D-loop instability (34.6%, 9 of 26).

Coding Genes/tRNAs

The results of the screening of mtDNA somatic mutations in coding genes, including *tRNAs* genes, are summarized in Tables 3 4, and 5. We detected 57 mtDNA somatic mutations in 34 of the 66 (51.5%) tumors (Table 3). These somatic mutations include three deletions [one of 15 bp in the *CytB* gene (patient 3) and two in the *COII* gene, one of 573 bp (patient 3) and another of 569 bp (patient 9)]; three *tRNA* somatic point mutations, one in the $tRNA^{Ser2}$ (patient 14), another in the $tRNA^{Ala}$ (patient 30), and another in $tRNA^{Ile}$ (patient 41); and 51 point mutations in the OXPHOS system genes (Table 3). From these 51 point mutations, 25 (49.0%) were silent mutations and 26 (51.0%) were missense mutations (Table 3); 44 of the 54 (81.5%) somatic point mutations (3 in *tRNAs* and 51 in OXPHOS system genes) were transitions. The technique we have used (automated direct sequencing) does not allow to decide with certainty if the mtDNA somatic mutations are homoplasmic or not, because it is not as sensitive as the manual sequencing. It is likely, however, that most somatic mutations are homoplasmic or near homoplasmic, because we have detected only a single signal in all but five cases in which two signals could be individualized. The same is also true for almost all mtDNA variants (see below). The results of the automated sequencing were confirmed in some samples using manual sequencing. The comparison of the prevalence of mutations in coding genes of the OXPHOS system in benign and malignant tumors did not yield any significant difference, but for the higher prevalence ($P = 0.015$) of missense point mutations in genes of the complex I in carcinomas than in adenomas (Table 4); the aforementioned missense mutations were detected in six of the seven genes of complex I (*ND1*, *ND3*, *ND4*, *ND4L*, *ND5*, and *ND6*) without any apparent concentration in a single gene (Table 3). The comparison of the prevalence of mutations in coding genes of the OXPHOS system in non-Hürthle cell and Hürthle cell tumors did not yield any significant differences (Table 5). There was however a trend ($P = 0.096$) toward a higher prevalence of missense point mutations in complex V genes in Hürthle cell tumors (Table 5); all of the missense mutations detected in complex V ($n = 4$) involved *ATPase 6* gene (Table 3).

Table 3. Summary of mtDNA Somatic Mutations in Sixty-Six Tumors

Case	Diagnostic	Age	Nucleotide position	Gene/region	Protein
1	PC	57	C15280T*	*CytB*/CpIII	Silent
3	HCFC	32	(14927–14941) del*	*CytB*/CpIII	5aa (TAFSS)
			(7631–8203) del*	*COII*/CpIV	191aa (16–206)
4	HCFC	39	A6650C*	*COI*/CpIV	Silent
			G9477T	*COIII*/CpIV	V91F
5	HCFC	33	T14498A*	*ND6*/CpI	Y59F
6	HCFC	76	G8697A	*ATPase6*/CpV	Silent
			A8701G	*ATPase6*/CpV	T59A
			C10272T*	*ND3*/CpI	$L_2$72F
7	HCPC	62	G9746A*	*COIII*/CpIV	Silent
8	HCA	60	G9477A	*COIII*/CpIV	V91I
9	HCA	49	A8701G	*ATPase6*/CpV	T59A
			(7627–8195) del*	*COII*/CpIV	Stop Codon
10A	PC	63	C4940T*	*ND2*/CpI	Silent
			T7785C*	*COII*/CpIV	I67T
10B	HCA	63	T9137C*	*ATPase6*/CpV	I204T
11	HCA	67	C12918T*	*ND5*/CpI	Silent
			C6473T*	*COI*/CpIV	Silent
12B	PC	45	C9030T*	*ATPase6*/CpV	Silent
			G9575C	*COIII*/CpIV	Silent
14	HCA	34	G12236A	$MTTS_2$	—
15	HCA	59	C7873T*	*COII*/CpIV	Silent
			G8697A	*ATPase6*/CpV	Silent
			A8706G*	*ATPase6*/CpV	Silent
16A	HCA	67	C7873T*	*COII*/CpIV	Silent
17	HCA	57	A15182G*	*CytB*/CpIII	I146V
			A4985G	*ND2*/CpI	Silent
18	HCA	38	A8716G*	*ATPase6*/CpV	K64E
20	HCPC	73	C11332T	*ND4*/CpI	Silent
			G7775A*	*COII*/CpIV	V64I
22	HCPC	46	G9655A	*COIII*/CpIV	S150N
23	HCFC	71	C10269T*	*ND3*/CpI	$L_2$71F
			G14560T*	*ND6*/CpI	Silent
25	HCPC	8	A4613G*	*ND2*/CpI	Silent
			G9477A	*COIII*/CpIV	V91I
27	HCFC	48	C3992T*	*ND1*/CpI	T229M
			C13943T*	*ND5*/CpI	T536M
28	A	42	C10793T*	*ND4*//CpI	$L_2$12L_1 (Silent)
30	A	54	C5633T	MTTA	—
			C7819A*	*COII*/CpIV	Silent
31	A	62	C9691T*	*COIII*/CpIV	A162V
			C7103T*	*COI*/CpIV	Silent
35	HCFC	69	G10197C*	*ND3*/CpI	A47P
40	HCA	77	G11016A*	*ND4*/CpI	$S_2$86N
			A10639G	*ND4L*/CpI	N57S_2
41	FC	40	C4312T	MTTI	—
			C10691G*	*ND4L*/CpI	Silent
44	FC	35	G3910A*	*ND1*/CpI	E202K
46	A	67	T15312G*	*CytB*/CpIII	I189S_2
51	PC	54	C3594T*	*ND1*/CpI	Silent
			G10320A*	*ND3*/CpI	V88I
			C11840T*	*ND4*/CpI	$L_2$361L_1 (Silent)
56	A	46	C9296T	*COIII*/CpIV	Silent
			C10181T	*ND3*/CpI	Silent
59	PC	40	G3526A*	*ND1*/CpI	A74T
			C13943T*	*ND5*/CpI	T536M
62	FC	54	A12967C*	*ND5*/CpI	T211P

*Unpublished sequence variants, (MITOMAP: A Human Mitochondrial Genome Database. Center for Molecular Medicine, Emory University, Atlanta, GA. *http://www.gen.emory.edu/mitomap.html, 2000*).

Mutations in D-Loop Region Versus *Prevalence of mtDNA CD and Mutations in Coding Genes*

The percentage of tumors with mtDNA CD was higher, although not significantly ($P = 0.148$), in the group of tumors with D-loop instability (78.1%, 25 of 32) than in the group without D-loop instability (61.8%, 21 of 34). The amount (mean ± SD) of mtDNA CD was significantly higher ($P = 0.045$) in tumors with D-loop instability (4.1 ± 3.2%) than in tumors without D-loop instability (2.6 ± 2.8%). The percentage of tumors with mtDNA somatic mutations was significantly higher ($P = 0.026$) in tumors with D-loop instability (65.6%, 21 of 32) than in tumors without D-loop instability (38.2%, 13 of 34).

Table 4. Summary of the mtDNA Somatic Point Mutations in Coding Genes of the OXPHOS System in Benign and Malignant Tumors

Diagnosis	CpI	CpIII	CpIV	CpV
Total point mutations				
Benign	5/30 (16.7%) $P = 0.077$	2/30 (6.7%) $P = 0.450$	7/30 (23.3%) $P = 0.700$	4/30 (13.3%) $P = 0.274$
Malignant	13/36 (36.1%)	1/36 (2.8%)	7/36 (19.4%)	2/36 (5.6 %)
Missense point mutations				
Benign	1/30 (3.3 %) $P = 0.015$	2/30 (6.7%) $P = 0.116$	2/30 (6.7 %) $P = 0.343$	3/30 (10.0%) $P = 0.221$
Malignant	9/36 (25.0%)	0/36 (0.0%)	5/36 (13.9%)	1/36 (2.8 %)

Cp, Complex of the OXPHOS system.

MtDNA Variants

The results of the study of mtDNA variants in the adjacent parenchyma of the tumors are summarized in Table 6. In the seven patients from whom blood samples were available the mtDNA variants detected in the normal adjacent parenchyma were also present in blood samples (data not shown); the mtDNA sequence variants detected in normal adjacent parenchyma were thus considered as germinal mtDNA variants.

We detected 253 sequence variants mtDNA in the normal parenchyma of the 59 patients; 112 of such mtDNA variants have not been previously published (data not shown). The distribution of the mtDNA variants by complex of the OXPHOS system is the following: complex I, 119; complex III, 26; complex IV, 60; and complex V, 48.

The comparison of the prevalence of mtDNA variants in patients with benign and malignant tumors shows a significant higher prevalence ($P = 0.001$ and $P = 0.005$, respectively) of complex I and complex IV variants in patients with malignant tumors than in patients with benign tumors (Table 6). Complex V variants were significantly more frequent ($P < 0.001$) in patients with Hürthle cell tumors than in patients with tumors without Hürthle cell features (Table 6).

Almost all of the variants detected in complex V genes of patients with Hürthle cell tumors involved the *ATPase 6* gene (91.9%, 34 of 37) and most of them were nonsilent (79.4%, 27 of 34). The same does not hold true regarding the variants detected in patients with non-Hürthle cell tumors [8 of 11 (72.7%) involved *ATPase6*; 2 (25.0%) were nonsilent and 6 (75.0%) were silent].

Discussion

The occurrence of mtDNA CD had been previously reported in Hürthle cell lesions of the thyroid[1,18,24,26] and in tumors of other organs,[16,17,25,26,30] namely those composed of oxyphilic cells such as the Warthin's tumor of the salivary glands.[25] The present study confirms the presence and abundance of mtDNA CD in Hürthle cell tumors regardless of their benign or malignant nature and their histotype. The mtDNA CD was also found, usually in extremely low levels, in the normal adjacent thyroid tissue of some tumors, thus supporting the concept that mtDNA CD precedes oxyphilic changes.[1,18,25,31]

The increased number of mitochondria and the mitochondrial structural abnormalities observed in Hürthle cell tumors mimic those detected in the cells of patients with several mitochondrial diseases and myopathies.[32–36] There is oxyphilic transformation of epithelial cells of the choroid plexus in mitochondrial encephalopathies (because of mtDNA alterations),[34] and oxyphilic changes associated to mtDNA alterations have been described in Leigh syndrome[35] and mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS).[36]

It has been suggested that mtDNA large deletions may reflect a deletion-prone region of mtDNA that is thought to be structural.[31,37] Such deletions would result in most instances from the action of reactive oxygen species over the mtDNA.[6,7] This assumption is supported by the results of the present study: 81.5% of the mtDNA somatic point mutations were transitions. The presence of mtDNA deletions in normal thyroid tissue may be the result of an

Table 5. Summary of the mtDNA Somatic Point Mutations in Coding Genes of the OXPHOS System in Non-Hürthle and Hürthle Cell Tumors

Diagnosis	CpI	CpIII	CpIV	CpV
Total point mutations				
Non-Hürthle	8/26 (30.8%) $P = 0.607$	2/26 (7.7%) $P = 0.322$	5/26 (19.2%) $P = 0.751$	1/26 (3.8 %) $P = 0.232$
Hürthle	10/40 (25.0%)	1/40 (2.5%)	9/40 (22.5%)	5/40 (12.5%)
Missense point mutations				
Non-Hürthle	4/26 (15.4%) $P = 0.966$	1/26 (3.8%) $P = 0.755$	2/26 (7.7 %) $P = 0.535$	0/26 (0.0 %) $P = 0.096$
Hürthle	6/40 (15.0%)	1/40 (2.5%)	5/40 (12.5%)	4/40 (10.0%)

Cp, Complex of the OXPHOS system.

Table 6. Number Per Case (Mean ± SD) of the mtDNA Variants in the Normal Adjacent Parenchyma of Patients with Benign and Malignant Thyroid Tumors and in the Normal Adjacent Parenchyma of Patients with Non-Hürthle and Hürthle Cell Thyroid Tumors, by OXPHOS System Complex

Diagnosis	Number of cases	Total variants mean ± SD		Complex I variants mean ± SD		Complex III variants mean ± SD		Complex IV variants mean ± SD		Complex V variants mean ± SD	
Benign	30	3.2 ± 1.6		1.4 ± 0.9		0.4 ± 0.5		0.6 ± 0.8		0.8 ± 0.6	
Malignant	36	4.4 ± 1.4	$P = 0.002$	2.2 ± 0.9	$P = 0.001$	0.4 ± 0.5	$P = 0.750$	1.1 ± 0.6	$P = 0.005$	0.7 ± 0.6	$P = 0.628$
Non-Hürthle	26	3.6 ± 1.4		1.9 ± 1.0		0.4 ± 0.5		0.9 ± 0.7		0.4 ± 0.5	
Hürthle	40	4.0 ± 1.7	$P = 0.378$	1.8 ± 1.0	$P = 0.601$	0.4 ± 0.5	$P = 0.557$	0.9 ± 0.8	$P = 0.830$	0.9 ± 0.6	$P < 0.001$

age-related accumulation effect of endogenous oxidative damage, aggression of lymphocytes in autoimmune thyroid diseases, or, as observed in some mitochondrial degenerative diseases, the result of alterations in a nuclear gene, or genes, affecting the mitochondrial biogenesis.[3,38,39] The oxidative pressure that results from tumor development can also produce reactive oxygen species and mtDNA damage in some lesions.[6–8,21]

The cause of mitochondrial proliferation in Hürthle cell tumors associated with mtDNA damage is unknown, although there is evidence of a decline in oxidative phosphorylation, and a higher requirement by the mitochondria for cytosolic ATP because of OXPHOS deficiency, which is accompanied by an induction of nuclear and mtDNA OXPHOS system gene transcriptions.[31] These findings suggest a defect in the energy production machinery of the cell and indicate that the increased mitochondrial content may be compensatory, putatively because of the involvement of nuclear genes controlling mitochondrial number through a sort of a feed-back mechanism.[40] Recently, Savagner and colleagues[41] showed that ATP production is deficient in a series of Hürthle cell tumors, mainly because of a coupling defect in mitochondria; this defect might be related to a twofold increase in UCP2 (uncoupling protein 2) expression.[41] Savagner and colleagues[41] suggested that mitochondrial proliferation could therefore be an adaptative response to the UCP2 overexpression or, alternatively, the UCP2 overexpression might be a response to the proliferation of mitochondria compensating for the decreased mitochondrial ATP synthesis because of OXPHOS abnormalities. The results we have obtained fit better with the latter hypothesis.

The trend to an association between D-loop instability and occurrence and amount of mtDNA CD may be a consequence of the mtDNA CD rather than its cause. Taking into consideration that the mtDNA CD leads to a higher replicative rate as a sort of compensatory effect,[34,42] it is possible that the instability in mtDNA may result from the high rate of mtDNA replication. Alternatively, it has been suggested that D-loop instability indicates the existence of a deficient repair mechanism of mtDNA alterations;[11,12,14] such deficiency might explain the concurrent existence of mtDNA point mutations and mtDNA large deletions in tumors with D-loop instability.[11,12,14] Most of the mtDNA point mutations are the result of mtDNA aggression by environmental factors, namely by reactive oxygen species,[6,7,21,43,44] whereas insertions/deletions could occur via a slipped replication mechanism.[45]

No mechanism related with nuclear mismatch repair genes has been found to be active in human mtDNA repair to date. In contrast with the results of Habano and colleagues[14] our data do not support the assumption that the instability in mitochondrial D-loop region is related with nMSI, because the level of mtDNA instability detected in the present study (48.5%) is much higher than the nMSI found in any type of thyroid tumor.[46–49] Furthermore, the histotypes with higher levels of mtDNA instability are those displaying Hürthle cell features in which nMSI was not detected.[49] Nuclear microsatellite instability was also not detected in any of the 12 cases in the present series that were analyzed previously (data not shown).

Our study documents a large number of somatic mtDNA mutations in all tumor histotypes. This high frequency is probably because of the high susceptibility of mtDNA to damage by mutagens.[5–8] Homoplasmic mtDNA mutations may reflect a replicative advantage for mutated mtDNA copies,[14,21,22] a growth advantage for a cell containing certain mtDNA mutations,[21,22] and/or tumorigenic properties of mtDNA mutations.[21,23] Coller and colleagues[50] and Jones and colleagues[51] have recently shown, by a mathematical approach, that these mutations could also arise by chance without any physiological advantage or tumorigenic requirement; this possibility is difficult to reconciliate with the occurrence of selection of mtDNA-specific variants in several disease models.[21,52–56]

The aforementioned hypotheses do not apply equally to silent and nonsilent mutations. The former, which represent almost half of the mtDNA mutations detected in our series (49%, 25 of 51), may have arisen by chance.[50,51] However, the same does not hold true for nonsilent mutations. The high frequency of mtDNA somatic mutations in the malignant tumors of our series, namely in complex I genes, suggests a role for these mutations in thyroid cancer. It has been advanced the presence of functionally relevant mtDNA mutations in some types of human tumors.[21,23,57] Cell fusion experiment showed that some mtDNA mutations, namely complex I mutations, are dominant, able to replace recipient mtDNA, and achieve homoplasmy in tissue culture quite rapidly.[21] These findings fit with the observation that most of the mtDNA somatic mutations described in human cancers are located in complex I genes.[14,22]

Yeh and colleagues[23] reported an association of complex I variants and homoplasmic mtDNA mutations and the occurrence of thyroid papillary carcinomas. Follicular and papillary carcinomas of our series carried a significantly higher prevalence of nonsilent mtDNA point mutations of complex I genes than adenomas. We also detected a significantly higher prevalence of complex I and complex IV sequence variants in the normal parenchyma adjacent to the malignant tumors. Our data thus support the hypothesis advanced by Yeh and colleagues[23] that the accumulation of some mtDNA variants and mtDNA somatic mutations seem to be involved in thyroid tumorigenesis.

Our results on the detection of mtDNA sequence variants suggest the existence of an association between germline polymorphisms of *ATPase 6* and the occurrence of Hürthle cell tumors. In man, complex V (ATP synthase) of the mitochondrion comprises 10 to 16 subunits encoded by nuclear DNA and two subunits (ATPase 6 and ATPase 8) encoded by mtDNA.[42] ATP synthase complex, at least in yeast, is involved in mtDNA genome maintenance and ATPase 6 seems to be one of the most important elements in this phenomenon.[39,58–60] In yeast, mutations that abrogate the synthesis of any of the ATPase subunits, even those that are unclearly encoded, cause the same phenotype, ie, high production of *petites variants* [variants having mtDNA deleted molecules (rho^-) or no mtDNA at all (rho^0)].[39,59]

The role of ATP synthase genes in mtDNA maintenance may contribute to explain our findings. It is tempting to suggest that, like in yeast, ATPase 6 may have a role in mtDNA maintenance in humans. The polymorphisms of *ATPase 6* could lead to a less efficient mtDNA replication and to mtDNA abnormalities that could contribute to the occurrence of mtDNA CD and Hürthle cell tumors. A larger series is needed to confirm this hypothesis and to find whether or not mutations in *ATPase 6* gene can be used as a tool to follow Hürthle cell transformation.

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